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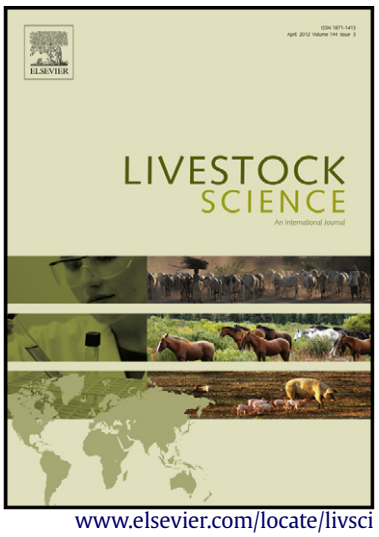

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For Livestock Science – invited paper

Title: Post-partum anoestrus in tropical beef cattle: a systems approach combining gene expression and genome-wide association results.

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Abstract

The length of the post-partum anoestrous interval affects reproductive efficiency in many tropical beef cattle herds. In this study, results from genome-wide association studies (*Experiment 1*: GWAS) and gene expression (*Experiment 2*: microarray) were combined in a systems approach to reveal genetic markers, genes and pathways underlying the physiology of post-partum anoestrus in tropically adapted cattle. The microarray study measured the expression of 13,964 genes in the hypothalamus of Brahman cows. A total of 366 genes were differentially expressed (DE) in the post-partum period, when acyclic cows were compared to cows that had resumed ovarian cycles. Associated markers ($P < 0.05$) from a high density GWAS pointed to 2,829 genes that were associated with post-partum anoestrous interval (PPAI) in two populations of beef cattle: Brahman and Tropical Composite. Together the experiments provided evidence for 63 genes that are likely to influence the resumption of ovulation post-partum in tropically adapted beef cattle. Functional annotation analysis revealed that some of the 63 genes have known roles in hormonal activity, energy balance and neuronal synapse plasticity. Polymorphisms within candidate genes identified by this systems approach could have biological significance in post-partum anoestrus and help select Zebu (*Bos indicus*) influenced cattle with genetic potential for shorter post-partum anoestrus.

Highlights

post-partum anoestrus length affects reproductive efficiency in tropical beef cattle

genome-wide association studies and gene expression results were combined in a systems approach

we found genes with changed expression in the brain that were also associated with anoestrus traits

these 63 genes could have biological significance in post-partum anoestrus in cows

these genes could also help select cattle with genetic potential for shorter post-partum anoestrus

Keywords

Zebu, Post-partum, Hypothalamus, Genomics, Microarray, Anoestrus, Fertility

Introduction

The period associated with the lack of ovulation after calving is termed post-partum anoestrus. An extended post-partum anoestrous period can be attributed to multiple causes (Yavas and Walton, 2000a, b). Major causes of failure to return to oestrus include the suckling stimulus, the energy demand of lactation and the presence of the calf (Acosta et al., 1983; Williams and Griffith, 1995). The calf imposes a strong negative influence on the resumption of ovulation and weaning has been used to induce ovulation in the post-partum period (Yavas and Walton, 2000a). Ovulation in response to weaning also depends on body condition, nutritional status, parity number and general health of the cow. Comparisons between suckled and weaned cows served as a model to study gene expression related to post-partum anoestrus (Flatscher-Bader et al., 2009; Ainu Husna et al., 2012). Gene expression differences between suckled and weaned cows are likely related to the resumption of oestrus cycles. Knowledge of these differences in gene expression serves to inform new strategies for management of post-partum anoestrus.

Prolonged post-partum anoestrus in tropical beef cattle is detrimental to reproductive efficiency. Tropical beef cattle usually have a high component of Zebu (*Bos indicus*)

ancestry, which is useful for adaptation purposes, including heat and parasite tolerance (Turner, 1972; Porto Neto et al., 2011; Burrow, 2012). However, Zebu cattle are prone to experiencing an extended post-partum anoestrous interval, which decreases reproductive rates and farm productivity. Management strategies have been suggested specifically for Zebu herds to improve reproductive performance (Baruselli et al., 2004; Sartori et al., 2010). Genetics also influences the length of the post-partum anoestrous interval. The heritability of the first post-partum anoestrus interval in a population of Brahman cows was estimated at 0.51 (Johnston et al., 2014). Thus, selective breeding and the use of associated polymorphisms were investigated as an approach to improve reproductive rates by shortening the anoestrous period (Hawken et al., 2012; Zhang et al 2014).

The aim of the current study was to identify genes, pathways and single nucleotide polymorphisms (SNP) associated with post-partum anoestrus in tropically adapted beef cattle. To identify associated SNP, genome-wide association studies (GWAS) were carried out in two populations of cattle, representing two tropically adapted breeds: Brahman and Tropical Composite. The genetics of Australian Brahman cattle are mostly Zebu, approximately 90% (Bolormaa et al., 2011). Tropical Composite is a term used to describe stable crosses between *Bos taurus* and Zebu breeds, which are typical of northern Australian herds (Barwick et al., 2009; Johnston et al., 2009). To discover differentially expressed genes, a microarray study was undertaken to compare suckled and weaned Brahman cows. The results from the two experimental approaches, GWAS and microarray, were integrated to strengthen the evidence for proposed candidate genes and pathways affecting post-partum anoestrus.

Materials And Methods

For the GWAS (*Experiment 1*), Animal Care and Use approval was not required because data analysed were obtained from existing phenotype and genotype databases of the

Cooperative Research Centre for Beef Genetic Technologies, as described in the following section. Approval for the gene expression study (*Experiment 2*) was granted by The University of Queensland Animal Ethics Committee (SAS/719/06/CRC).

***Experiment 1*: Genome-wide association studies**

Animals and phenotypes

Brahman (n = 843) and Tropical Composite (n = 866) cows were part of a larger genetics study, detailed previously (Johnston et al., 2009; Johnston et al., 2014). The phenotype targeted in the current study was post-partum anoestrous interval (PPAI, days). Cows were two years old on average at their first breeding season. After first calving, PPAI was measured as the number of days from calving to first ovulation post-partum (also termed first re-breeding interval). Ovarian scans were performed every two weeks to determine the time of ovulation (detection of *corpus luteum*).

Genotypes

For genotyping, DNA was extracted from blood samples of each cow. The BovineSNP50 bead chip version 1 (Matukumalli et al., 2009) was used to genotype all cows, as described previously (Hawken et al., 2012). In brief, SNP chips were processed according to the manufacturer's protocols (Illumina Inc., San Diego, CA), repeated samples were included in the genotyping for quality assurance and the Bead Studio software (Illumina Inc., San Diego, CA) was used to determine genotype calls. Genotype calls were coded as 0 for the homozygote of the A allele, 1 for the heterozygote, and 2 for the homozygote of the B allele. Alleles A and B were defined according to top rule from Illumina.

In addition, sires and population representative animals (917 DNA samples) were genotyped with the high-density BovineHD SNP chip (~770,000 SNP; Illumina Inc., San Diego, CA) to allow for genotypic imputation. Complete high-density genotypes were imputed using the BEAGLE 3.2 program (Browning and Browning, 2010) for all samples. Imputation was carried out within breeds, as detailed previously (Fortes et al., 2013b).

Association analysis

Association analyses were carried out separately for each breed. The additive effect of each SNP on PPAI was calculated by regression analysis, with genotypes coded as 0, 1, or 2 copies of the B allele, and fitting the following mixed model:

$$y_i = X\beta + Zu + s_k + e_i \quad (1)$$

where y_i represents the vector of observations from the i^{th} cow, X is the incidence matrix relating fixed effects in β with observation in y_i, Z is the incidence matrix relating random additive polygenic effects in u with observation in y_i, s_k represents the additive association of the k^{th} SNP, and e_i is the vector of random residual effects. Fixed effects included in β were discussed previously (Johnston et al., 2009; Johnston et al., 2014). Polygenic effects were included to reduce the effect of family structure. Over 50 sire families were represented in each breed.

Standard stochastic assumptions applied to the random effects in model (1), which were assumed to be distributed as multivariate normal with zero mean and variance as follows:

$$\mathrm{V}\begin{bmatrix} u \\ e \end{bmatrix} = \begin{bmatrix} A\sigma_u^2 & 0 \\ 0 & I\sigma_e^2 \end{bmatrix} \quad (2)$$

where A is the numerator relationship matrix across all cows and derived from the pedigree structure (Wright, 1922); σ_u is the additive polygenic component of variance; I is an identity

matrix; and σ_e is the residual component of variance. To perform GWAS analysis Qxpack software was used (Perez-Enciso and Misztal, 2011).

***Experiment 2*: Gene expression**

Animals and phenotypes

Three-year-old Brahman cows that had calved for the first time were used for this trial. In the post-partum period, the cows either continued to suckle a calf (n = 4) or were weaned (n = 4). The latter cows were separated from their calves between 20 and 37 days after calving and slaughtered 13 days after weaning. Weaned cows were slaughtered on the same day as suckled cows, matched by number of days post-partum. The suckled cows were separated from their calves for transport to the abattoir (18 to 24 h before slaughter). A related study reported quantitative real time polymerase chain reaction (qRT-PCR) results for candidate genes, using this same animal resource (Ainu Husna et al., 2012).

Ovarian phenotype was examined at the time of slaughter. Suckled cows had supressed ovarian follicular growth with a maximum follicle size of 5 mm (n = 4) and weaned cows had a *corpus haemorrhagicum* or *corpus luteum* indicating that they had resumed ovulation (n = 4).

Tissue sampling and RNA extraction

Cows were slaughtered using a non-penetrating captive bolt. Brains were initially sectioned using a medial sagittal incision to reveal the hypothalamus. The hypothalamic region on each side of the brain was dissected into 2 sub-regions classified as H1 and H2, as detailed previously (Ainu Husna et al., 2012). Within 90 minutes, approximately 0.4 g of brain sample was collected and stored in RNAlater (Qiagen, www.qiagen.com) until RNA

extraction. Total RNA was extracted using a modified RNeasy extraction kit (Qiagen, www.qiagen.com) protocol. RNA integrity number (RIN) was verified using the Bioanalyzer (Agilent Technologies, http://www.home.agilent.com). Residual Genomic DNA contamination was removed with the DNA free kit (Ambion, http://www.lifetechnologies.com/au/en/home/brands/ambion.html).

Microarray

For microarray, an aliquot of 500 ng of each RNA sample was reverse-transcribed. Samples were amplified and either labelled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5) by T7-polymerase in vitro transcription, to obtain fluorescent-labelled cRNA using the low RNA input Linear Amplification Kit from Agilent Technologies (http://www.home.agilent.com). The Agilent Bovine-Four-Plex G2519f DNA oligonucleotide microarray (Agilent Technologies) was the platform used. This microarray platform contained 21,475 unique 60-mer probes representing approximately 19,500 distinct bovine genes (sequences and probe annotations are available at http://www.chem.agilent.com/). For improved functional annotations and identities of probes, all the Agilent probes were re-annotated as follows: 1) all probes were annotated with direct BLAST searches (http://www.ncbi.nlm.nih.gov/) and 2) all probes were aligned to the human and bovine reference sequences (RefSeq) collection mapped on the bovine genome assembly (UMD3) All probes were further tested for overlaps or close proximity to bovine RefSeq. These procedures identified 13,964 bovine protein-coding genes corresponding to the microarray probes.

Microarrays were hybridised according to standard protocol at the SRC Microarray Facility of the Institute for Molecular Biosciences, in Brisbane, Australia (http://microarray.imb.uq.edu.au). In brief, hybridisation was conducted in an Agilent DNA Microarray Hybridisation Oven using Gene Expression Hybridisation Kit and Wash Buffer

Kits from Agilent Technologies according to the manufacturer's instructions. Arrays were placed in Agilent Stabilisation and Drying Solution prior to scanning on an Agilent G2565AA scanner. Raw data processing was performed using Agilent Feature Extraction Software. Quality control measures of image analyses were performed: spot quality, normalisation against background, reproducibility, uniformity and sensitivity.

Statistical analysis and differentially expressed genes

For the analysis presented here, suckled cows with a suppressed ovarian phenotype (n = 4) were compared to weaned cows that had ovulated (n = 4). Hypothalamus regions H1 and H2 were treated as separate tissue samples for RNA extraction and microarray analysis. However, for the statistical analysis presented here, gene expression values of the 2 regions were merged to represent overall hypothalamic gene expression.

Mixed model analyses were used for gene expression data normalisation (Reverter et al., 2005). Differentially expressed genes were identified by model-based clustering by a mixture of distributions on the normalised expression data of each gene at each condition: weaned versus suckled, following established methods (Reverter et al., 2004; Reverter et al., 2005). The mixed model was fitted to the intensity readings as below:

$$Y_{ijkhgn} = \mu + C_{ijk} + G_g + AG_{ijg} + DG_{kg} + VG_{hg} + \varepsilon_{ijkhgn} \qquad (1)$$

where Y_{ijkhgn} represents the n^{-th} background-adjusted, normalised base-2 log-intensity from the g^{-th} gene (or probe) at the h^{-th} phenotype variety (suckled or weaned), from the i^{-th} chip, j^{-th} array (there are four microarrays per chip) and k^{-th} dye channel; μ is the overall mean; C represents a comparison fixed group effects with 64 levels and defined as those intensity measurements from the same chip, array and dye channel; G represents the random gene (or probe) effects with 21,475 levels; AG, DG, and VG are the random interaction effects of

array by gene, dye by gene, and variety by gene, respectively; and finally, ε is the random error term. For the random effects in Model (1), standard stochastic assumptions are:

$$G \sim \text{iid } N(0, \sigma^2_g),$$

$$AG \sim \text{iid } N(0, \sigma^2_{ag}),$$

$$DG \sim \text{iid } N(0, \sigma^2_{dg}),$$

$$VG \sim \text{iid } N(0, \sigma^2_{hg}),$$

and $$\varepsilon \sim \text{iid } N(0, \sigma^2_e),$$

where iid denotes independently and identically distributed and N denotes the normal distribution. Variance components are between genes (σ^2_g), between genes within array (σ^2_{ag}), between genes within dye (σ^2_{dg}), between genes within treatment (σ^2_{hg}), and within genes (σ^2_e). Variance components were estimated using restricted (to zero error contrasts) maximum likelihood (Searle et al., 1992).

To contrast the expression of each gene across the two varieties (suckled and weaned), we explored the following measure of differential expression:

$$d_i = VG_i^{WEANED} - VG_i^{SUCKLED}$$

Large positive (or large negative) values of d_i are likely to indicate that the *i*-th gene is up-regulated (or down-regulated) in the weaned samples. Using a nominal P-value < 0.01 from a two-tailed t-test statistic, genes were deemed to be DE if their normalized measure of differential expression fell beyond 2.57 standard deviations..

The resulting list of DE genes was used as the target gene list compared to the background gene list (all bovine annotated genes) in gene ontology (GO) enrichment analysis, performed using GOrilla (Eden et al., 2009). Functional annotation analyses and search for transcription factors that would target the DE genes were also performed for this target gene list, using DAVID (Dennis et al., 2003; Huang da et al., 2009).

Systems approach

The objective of this analysis was to integrate results from *Experiments 1* and *2* and identify genes that were related to post-partum anoestrus according to both approaches (GWAS and microarray). The systems approach followed two procedures: 1) having the GWAS as a starting point and 2) having the microarray as a starting point.

Having the GWAS as a starting point meant looking at the location of associated SNP to identify candidate genes. First, SNP associated to PPAI in both breeds were selected at two significance thresholds, $P < 0.05$ and $P < 0.01$. The genes closest to these SNP formed the lists of candidate genes from GWAS. Second, the list of DE genes from *Experiment 2* was searched for the candidate genes from GWAS to create a list of genes in common.

Having the microarray as a starting point meant searching the vicinity of the DE genes for associated SNP in the GWAS. All SNP located within 2,500 base pairs of each DE gene were checked for their association ($P < 0.05$ and $P < 0.01$) with PPAI in Brahman and in Tropical Composite cattle. To do this exercise, an additional annotation step was require in which all DE genes were mapped to UMD3.1 Bovine genome assembly, using four sources of gene annotation: Ensembl, UCSC, NCBI, and UCSC Xeno. Gene annotation resources were prioritized in that order, with the first priority as the Ensembl genes because these are manually curated, and therefore should represent the highest quality of annotation.

The list of candidate genes generated by integrating SNP association and gene expression data was used as a target gene list for functional annotation using GOrilla (Eden et al., 2009) and DAVID (Dennis et al., 2003; Huang da et al., 2009). Functional analyses searched for enriched gene ontology terms, pathways and transcription factors that would target the candidate genes.

RESULTS

Experiment 1: Genome-wide association studies

Manhattan plots summarizing SNP associations show chromosomes 21 and X as harbouring the most prominent results for Brahman and chromosome 5 for Tropical Composite (Figure 1). A total of 8,424 SNP were associated (uncorrected $P < 0.05$) with PPAI in both breeds. At a more stringent association level (uncorrected $P < 0.01$) 426 SNP were associated with PPAI in both breeds. All chromosomes had SNP associations in the first comparison between breeds (uncorrected $P < 0.05$). Except for chromosomes 25 and 28, all chromosomes had SNP associated with PPAI at the more stringent level (uncorrected $P < 0.01$) as shown in Table 1 (detailed list of associated SNP available from authors upon request). Associations within breed were not corrected for multiple testing and the above mentioned are uncorrected *P*-values. Correction for false discoveries is based on the validation of these SNP in two independent cattle populations, represented by the two different breeds. Only SNP that were significant (uncorrected $P < 0.05$) in both breeds were considered for the integrated analysis.

Experiment 2: Gene expression

A total of 366 genes were DE in the hypothalamus of weaned cows that had ovulated when compared to suckled cows that were in post-partum anoestrus (Supplementary Table S1). This list of DE genes was used as the target gene list in the GO enrichment analysis. The DE list was enriched for the GO biological function term hormone activity (GO:0005179, $P = 2.19 \times 10^{-2}$, corrected for multiple testing). The DE genes associated to hormone activity were: *EDN2* (endothelin 2),*GHRH* (growth hormone releasing hormone), *AGRP* (agouti related protein homolog, mouse), *IGF2* (insulin-like growth factor 2), *ADM*

(adrenomedullin), *ADCYAP1* (adenylate cyclase activating polypeptide 1, pituitary), *NPY* (neuropeptide y), *CALCB* (calcitonin-related polypeptide beta), *GH1* (growth hormone 1), *CCK* (cholecystokinin), and *C1QTNF9* (c1q and tumor necrosis factor related protein 9). Given the biological function similarities between *ADCYAP1* and *GNRH1*; *ADCYAP1* is discussed in more detail, despite not harbouring SNP that were associated with PPAI in both breeds (Table S3). Functional analysis revealed no enrichment for any known pathway in this list of 366 DE genes. However, the transcription factor *HNF1* was included in this gene list ($P = 7.7 \times 10^{-4}$, corrected for multiple testing).

Systems approach

In total, 56 genes were DE and were the nearest genes from SNP associated ($P < 0.05$) with PPAI in both breeds (Supplementary Table S2). Only 3 genes, *ALDH1A2*, *SLCO1C1*, and *SLITRK6,* were DE and were located near SNP associated with PPAI at the higher level of $P < 0.01$, in both breeds. No specific pathway was enriched when considering these 56 DE genes for functional analysis. However, 6 of these 56 genes have known biological functions related to neuronal activity and it is possible to hypothesize their roles in the context of post-partum anoestrus (see discussion referring to *MATN2, PLEKHF1*, *SLITRK6*, *SYT1*, *SYT4* and *WIF1*). The 56 genes were targets for a total of 97 transcription factors ($P < 0.05$, corrected for multiple testing, Supplementary Table S4).

From the 366 DE genes, 39 were located within 2,500 bp of SNP associated ($P < 0.05$) with PPAI in both breeds. Most of these genes were already captured in the first filtering procedure described above. The few exceptions were: *CPM*, *LOC100848886*, *MYO5B*, *OCA2*, *RARB*, *SERPINI2*, and *WBSCR17*. Together, both filtering procedures yield a list of 63 genes that are likely to influence resumption of ovulation post-partum in cattle, as evident from both GWAS and gene expression data. The number of SNP associated ($P <$

0.05) with PPAI neighbouring these 63 genes, the average *P*-value for SNP association in Brahman and Tropical composite cattle, and the normalized DE of each gene is presented in Table 2.

Functional analysis in DAVID using the 63 candidate gene list resulted in enrichment for the ontology term "neuron projection morphogenesis" (*P*-values: 6.2×10^{-4}, raw, and 9.4×10^{-2}, corrected for multiple testing). Genes underpinning this ontology term were: *PTK2*, *SLITRK6*, *CTNNA2*, *EFNA5*, *GBX2*, and *PRKCA*. Also, a total of 107 transcription factors were significant ($P < 0.05$, corrected for multiple testing) for these 63 candidate genes (Supplementary Table S5). The lists of transcription factors associated with the initial 56 candidate genes and these 63 candidate genes are quite similar, with *LHX3* being the top transcription factor in both (Supplementary Tables S4 and S5).

Of note, when mapping the 366 DE genes, 10 genes were located more than 2,500 bp away from any SNP in the HD chip. These genes were *URM1, ZBTB34, CDK10, BEX4, LOC780876, MBLAC1, PLA2G2D1, LOC100336726,* and *LOC506823*. The gene to SNP distance for these genes implies that they might not be captured in the GWAS experiment and were therefore not covered by the integrated analysis. To identify associated SNP tagging these 10 genes, the development of target assays would be required, but this is beyond the current study.

DISCUSSION

In this study, results from GWAS and gene expression studies were integrated in a systems analysis that revealed genetic markers, genes and transcription factors underlying the physiology of post-partum anoestrus in tropically adapted cattle.

The high-density GWAS largely confirms genomic regions associated with PPAI in the previous 50,000 SNP experiment performed with the same populations (Hawken et al.,

2012). Differences in SNP associations between Brahman and Tropical Composite cattle remained important, with a relatively small number of SNP being associated with PPAI in both breeds (8,424 SNP, $P < 0.05$; 427 SNP, $P < 0.01$). The fact that associated SNP are distributed across the genome confirms the complex inheritance of PPAI discussed before (Hawken et al., 2012).

Hormonal activity in the hypothalamus and pituitary gland is fundamental for the resumption of ovulation post-parturition (Yavas and Walton, 2000b). Gene ontology analysis of the 366 DE genes confirmed the importance of hormonal activity in our dataset. "Hormonal activity" was the only ontology term for which significant enrichment could be demonstrated in this set of 366 genes. The group of 11 genes underpinning this enrichment cluster consisted mainly of neuropeptides with roles in the regulation of lactation, appetite and energy balance. While potential roles for these neuropeptides in the hypothalamus of the post-partum cow are plausible, based on their known functions, the precise nature of their involvement has not been documented.

Bovine lactation is under the control of growth hormone, and SNP variation in *GH1* has been found to be associated with lactation and fertility traits in dairy cattle (Mullen et al., 2011). While *GHRH* is known to be synthesised and secreted by hypothalamic neurons, the expression of *GH1* in the hypothalamus was unexpected. The classical site for *GH1* expression is the anterior pituitary. The reason for *IGF2* differential expression in bovine hypothalamus is likewise unknown. *IGF2* has neurotrophic functions in neural tissue and the hypothalamus contains *IGF1R*-bearing cells capable of responding to this growth factor (Russo et al., 2005). The association of genes in the *IGF1* pathway with reproductive traits in cows has been discussed (Fortes et al., 2013a). A recent study reported the association between a SNP in the *IGF2* vicinity and pregnancy status in Brahman cattle (Reverter et al., 2014). *AGRP*, *CCK* and *NPY* code for hypothalamic neuropeptides with well-characterised

roles in metabolic regulation, and their inclusion in the DE list could reflect the important impacts of lactation on appetite and energy balance. *CALCB* and *ADM* belong to a family of neuropeptides with overlapping receptor affinities and biological effects that are important in maintaining normal placental function (Chauhan et al., 2009). The role of these "Hormonal activity" genes in the hypothalamus of the post-partum cow is yet unclear and merits further investigation.

The gene that codes for GnRH, *GNRH1*, is notable by its absence from the list of DE hypothalamic genes and from our GWAS results, as it has a well-known role as the master regulator of gonadotropin release from the pituitary (Bliss et al., 2010). The microarray assay used had no probes for *GNRH1,* which was a practical limitation for the study. We confirmed the lack of differential RNA expression of *GNRH1* by qRT-PCR (data not shown). However, we detected the differential gene expression of *ADCYAP1*. Similarly to GnRH, the pituitary adenylate cyclase-activating polypeptide encoded by ADCYAP1 is also is secreted by hypothalamic neurons into the hypophyseal-pituitary portal vasculature and regulates gonadotropin gene expression (Thomas et al., 2013). It is tempting to speculate that in the beef cows studied here, differential expression of *ADCYAP1*during the post-partum period is an important and so far overlooked driver of increased ovarian follicular activity and ovulation.

The 63 genes that were identified for a possible role in post-partum anoestrus both by GWAS and gene expression microarray could represent an important shortlist of molecules with functional roles in the resumption of ovulation in cows. Enrichment for the ontology term "neuron projection morphogenesis" was detected when using these genes as the target list. Modifications in axons, synapses and neuronal pathways are components of the neuronal plasticity that underpins changes in hypothalamic hormonal activity in the post-partum period. The function of 11 of the 63 genes that emerged from this integrated analysis could be

associated with neuronal plasticity (*MATN2, PLEKHF1, SLITRK6, SYT1, SYT4, WIF1, PTK2, CTNNA2, EFNA5, GBX2,* and *PRKCA*). Failure of *MATN2* protein up-regulation in knockout mice resulted in impaired axon regeneration (Malin et al., 2009). A role for *MATN2* in regenerating cattle hypothalamic function in the post-partum is a novel hypothesis emerging from our results. *PLEKHF1* was identified as one of 5 glucocorticoid responsive genes in rat hypothalamus (Sato et al., 2008). A deficiency in *SLITRK6* was associated with delay in synaptogenesis that impacts vision and hearing in mice and humans (Tekin et al., 2013). Our results suggest that *PLEKHF1* and *SLITRK6* may have a role in hypothalamic synapses and this warrants further investigation. Hypothalamic expression of *SYT1* and *SYT4* was associated with steroid hormonal variations of the oestrous cycle in mice (Crispino et al., 1999). Further, *SYT1* and *SYT4* encode synaptic vesicle membrane proteins involved in neurotransmitter and peptide secretion from small secretory vesicles (Xi et al., 1999). Importantly, *SYT4* regulates oxytocin exocytosis (Zhang et al., 2011). This regulation of oxytocin activity is likely to impact on post-partum anoestrus in cattle, considering our results and the extensive literature linking oxytocin with lactation and anoestrus (Williams and Griffith, 1995; Griffith and Williams, 1996). Wnt signalling, including the expression of its inhibitor *WIF1*, is important for neurogenesis in the hypothalamus of zebrafish throughout their lifecycle (Wang et al., 2009). Our results suggest that *WIF1* may also contribute to neuronal remodelling in the hypothalamus of post-partum cows, a novel hypothesis.

The lists of transcription factors associated with the candidate genes from the two filtering procedures are quite similar. This is expected since there was significant overlap between the two lists. Importantly, the second filtering procedure resulted in the addition of *ER* and *P53* to the list of transcription factors associated to post-partum anoestrus. Both *ER* and *P53* have important reported biological functions in female reproductive physiology

(Tasende et al., 2002; Hu, 2009; Micevych and Christensen, 2012; Micevych and Kelly, 2012).

In conclusion, microarray and GWAS studies together provide complementary evidence for genes, transcription factors and SNP that are likely to influence ovarian follicular activity and ovulation in post-partum Zebu and Zebu-crossed beef cattle. Disruptive polymorphisms within these gene regions could assist genomic selection predictions, with the aim to shorten post-partum anoestrus and improve reproductive performance. Future studies are likely to target genes reported here to discover putative causative mutations that could contribute to genomic selection accuracy in Zebu influenced breeds.

Conflict of interest

Authors acknowledge that this research was funded by CSIRO, The University of Queensland and Meat and Livestock Australia. These research institutions were collaborators in the Cooperative Research Centre for Beef Genetic Technologies, which provided data for the reported research. The authors declare no other conflict of interest.

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Figure title.

Figure 1. Manhattan plots for genome-wide association studies performed in Brahman cattle (A) and in Tropical Composite cattle (B).

Tables

Table 1. Number of single nucleotide polymorphisms (SNP) associated with post-partum anoestrus interval per chromosome, in two cattle breeds, Brahman and Tropical Composite, at two *P*-value thresholds.

Chromosomes	SNP ($P < 0.05$)	SNP ($P < 0.01$)
1	347	7
2	301	30
3	339	2
4	238	10
5	714	67
6	417	16
7	227	2
8	429	19
9	254	8
10	330	16
11	349	18
12	171	12
13	433	40
14	571	30
15	242	21
16	392	27
17	323	19
18	171	4
19	161	14
20	119	2
21	381	14
22	132	3
23	171	9
24	170	4
25	61	0
26	98	1
27	184	11
28	78	0
29	134	5
X	487	15
Total	8424	426

Table 2. Candidate genes related to post-partum anoestrus: evidence from GWAS and gene expression analysis.

Gene	BTA	Gene start (Mbp)	n SNP*	Average *P*-value		DE**
				Brahman	Tropical Composite	
ABI3BP	1	45.67	2	3.52×10^{-2}	1.91×10^{-2}	-3.23
ACBD6	16	62.93	2	3.04×10^{-2}	3.87×10^{-3}	-2.61
ACCN1	19	16.35	4	4.09×10^{-2}	3.09×10^{-2}	-2.83
ADAMTS20	5	37.10	3	3.62×10^{-2}	9.12×10^{-3}	-3.73
ADH6	6	26.79	1	2.45×10^{-2}	4.48×10^{-2}	2.53
ALDH1A2	10	52.34	1	6.06×10^{-3}	8.85×10^{-3}	2.28
ARHGAP21	13	25.73	1	3.25×10^{-2}	5.00×10^{-2}	2.75
CA12	10	46.75	1	4.12×10^{-3}	2.20×10^{-2}	-3.11
CASP1	15	3.46	4	4.46×10^{-2}	4.12×10^{-2}	2.54
CLN6	10	15.03	1	2.28×10^{-2}	1.65×10^{-2}	-3.31
COPS8	3	117.18	2	1.61×10^{-2}	3.28×10^{-2}	-2.63
CPM	5	45.08	1	2.00×10^{-2}	1.78×10^{-2}	3.86
CSRP1	16	49.33	2	2.13×10^{-2}	1.93×10^{-2}	-4.79
CTNNA2	11	54.72	10	2.87×10^{-2}	1.62×10^{-2}	-2.9
DCAF12	8	76.83	5	4.91×10^{-5}	3.51×10^{-2}	2.42
EFNA5	7	109.05	1	1.33×10^{-2}	3.30×10^{-2}	-2.49
EML1	21	66.36	1	1.96×10^{-2}	4.22×10^{-2}	2.47
ESR1	9	89.97	1	4.65×10^{-2}	1.38×10^{-2}	-3.14
FOXA1	21	48.21	1	2.50×10^{-2}	3.80×10^{-2}	2.48
GBX2	3	116.27	2	2.70×10^{-2}	2.97×10^{-2}	-4.1
GTPBP8	1	58.15	3	6.37×10^{-2}	3.82×10^{-2}	-2.54
HECTD2	26	13.06	1	2.93×10^{-2}	2.32×10^{-2}	-3.94
HIVEP1	23	44.14	1	2.28×10^{-2}	4.41×10^{-2}	-5.08
HS2ST1	3	57.11	1	2.35×10^{-2}	4.31×10^{-2}	-2.52
ITFG1	18	15.63	11	2.85×10^{-2}	1.26×10^{-2}	-2.52
KITLG	5	18.32	1	4.11×10^{-2}	3.39×10^{-2}	-3.14
LAMA4	9	38.64	3	2.65×10^{-2}	3.94×10^{-2}	-3.38
LIG1	18	55.31	5	2.22×10^{-2}	1.02×10^{-2}	-2.38
LOC100848886	21	29.75	2	3.32×10^{-2}	1.09×10^{-2}	-2.86
MAPK10	6	102.69	3	3.96×10^{-2}	3.59×10^{-2}	-2.53
MATN2	14	68.48	1	4.17×10^{-2}	2.63×10^{-2}	2.98

MPP7	13	36.61	3	7.01×10^{-3}	1.49×10^{-2}	-2.48
MYO5B	24	49.96	1	4.56×10^{-2}	3.08×10^{-2}	-2.47
MYOM3	2	129.39	1	4.40×10^{-3}	2.55×10^{-2}	-3.48
NFATC2	13	80.02	1	2.68×10^{-2}	3.61×10^{-2}	2.46
NUP188	11	99.46	1	3.17×10^{-2}	2.69×10^{-2}	-3.92
OCA2	2	0.35	1	2.07×10^{-2}	1.22×10^{-2}	-2.51
PAX8	11	46.78	3	3.91×10^{-2}	2.56×10^{-2}	2.72
PCDH7	6	51.54	1	3.20×10^{-2}	2.03×10^{-2}	-2.41
PDE6B	6	108.82	1	7.94×10^{-3}	3.22×10^{-2}	-4.22
PITRM1	13	45.47	1	4.27×10^{-2}	4.82×10^{-2}	4.04
PLA2G2D1	2	133.18	1	3.52×10^{-2}	1.54×10^{-2}	-2.98
PLEKHF1	18	40.42	1	3.26×10^{-2}	2.67×10^{-2}	2.33
PLEKHH1	10	79.77	1	7.30×10^{-3}	2.57×10^{-2}	-2.71
PRDM10	29	36.68	1	2.13×10^{-2}	3.68×10^{-2}	-2.39
PRKCA	19	63.28	1	1.60×10^{-3}	4.86×10^{-2}	-3.1
PSTPIP1	21	32.64	1	4.09×10^{-2}	2.15×10^{-2}	-2.45
PTK2	14	3.87	2	3.96×10^{-2}	1.02×10^{-2}	-3.54
RARB	27	40.13	2	3.41×10^{-2}	3.62×10^{-2}	-2.88
SERPINI2	1	100.79	2	4.60×10^{-3}	3.51×10^{-2}	-3.61
SGCA	19	37.11	1	3.60×10^{-2}	3.00×10^{-2}	2.68
SLC44A1	8	96.77	1	4.37×10^{-2}	2.32×10^{-2}	2.26
SLCO1C1	5	89.44	5	1.93×10^{-2}	6.16×10^{-3}	-3.04
SLITRK6	12	61.06	7	1.91×10^{-2}	2.86×10^{-2}	-2.46
SP140	2	118.93	1	2.85×10^{-2}	2.59×10^{-2}	2.5
STAC	22	10.16	2	2.30×10^{-2}	1.70×10^{-2}	-2.64
SYT1	5	8.84	1	4.99×10^{-2}	1.87×10^{-2}	-3.54
SYT4	24	12.95	1	3.26×10^{-2}	2.57×10^{-2}	-2.65
TRPA1	14	37.72	2	2.63×10^{-2}	2.96×10^{-2}	-5.09
VIT	11	19.18	2	2.24×10^{-2}	3.69×10^{-2}	3.02
WBSCR17	25	29.39	1	3.09×10^{-2}	1.37×10^{-2}	-3.8
WIF1	5	48.92	5	3.71×10^{-2}	5.38×10^{-3}	-3.3
ZWINT	26	2.85	1	8.83×10^{-4}	4.74×10^{-2}	2.36

*n SNP: number of single nucleotide polymorphisms within 2.5Kbp from the gene and associated ($P < 0.05$) in both breeds.

**DE: normalized differential expression from the contrast "Weaned minus Suckled" expressed in the base-2 log-scale. For instance, the -3.23 down-regulation of ABI3BP implies a 9.4 fold change (ie. from 2 to the power 3.23).

Figure

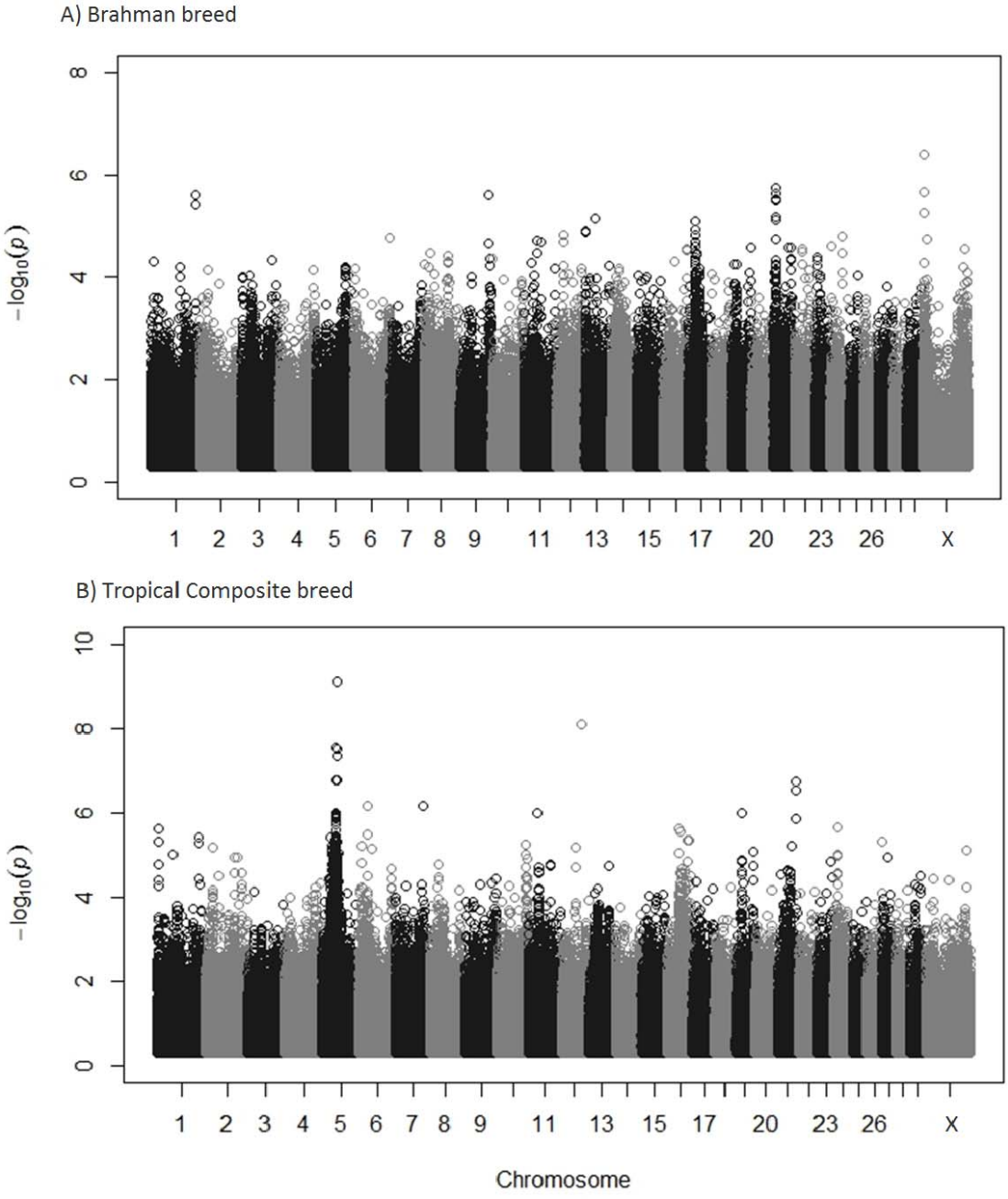

Figure 1. Manhattan plots for genome-wide association studies performed in Brahman cattle (A) and in Tropical Composite cattle (B).